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(54) Enhanced expression of proteolytic enzymes in koji mold

(57) The present invention has for object a koji mold which is capable to express at least 2 times more endo- and exo-peptidases than the wild type strain *Aspergillus oryzae* CNCM I-1882, and especially at least 30 mU of endopeptidase activity, at least 30 mU of leucine-amino-peptidase activity and at least 10 mU of prolyl-dipeptidyl-peptidase activity per ml of supernatant when grown in a minimal medium containing 0.2% soy bean proteins. The invention also provides a DNA-binding protein of *Aspergillus oryzae* (AREA) having at least the amino-acid sequence from amino-acid 1 to amino-acid 731 of SEQ ID NO:2 or functional derivatives thereof. The invention also provides a DNA molecule that comprises an *are*A gene encoding the DNA-binding protein according to the invention. In a fourth aspect, the invention provides a method for over-producing proteolytic enzymes, comprising cultivating a koji mold according to the invention in a suitable growth medium under conditions that the mold expresses enzymes, and optionally isolating the enzymes in the form of a concentrate. In another aspect, the invention provides the use of the koji mold of the invention to hydrolyze protein-containing materials. In a last further aspect, the invention provides a food product comprising a protein hydrolysate obtainable by fermentation with a koji mold of the invention of a material comprising proteins and at least 5mM of L-glutamine.

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Description

[0001] The invention relates to genetic modifications of koji molds allowing enhanced expression of proteolytic enzymes.

State of the art

[0002] Hydrolyzed proteins, which are widely used in the food industry, may be prepared by hydrolysis of protein material with acid, alkali or enzymes. Various methods have been used koji molds for the preparation food products, which are hydrolyzed by action of a large variety of secreted amylases, proteinases and peptidases. Koji molds are those traditionally used for making a koji culture (US4308284) including cells of the genus *Aspergillus*, *Rhizopus* and/or *Mucor*, especially *Aspergillus soyae*, *Aspergillus oryzae*, *Aspergillus phoenicis*, *Aspergillus niger*, *Aspergillus awamori*, *Rhizopus oryzae*, *Rhizopus oligosporus*, *Rhizopus japonicus*, *Rhizopus formosaensis*, *Mucor circinelloides*, *Mucor japanicus*, *Penicillium glaucum* and *Penicillium fuscum*, for example.

[0003] According to the rules of the International Code of Botanical Nomenclature (ICBN), *Aspergillus* is an anamorphic genus. This means that true Aspergilli only reproduce asexually through conidiophores. However, the typical *Aspergillus* conidiophore morphology can also be found in fungi that can reproduce sexually via ascospores. Some *Aspergillus* taxonomists caused confusion, because they did not adhere to ICBN terminology. Instead, they attempted to make various revisions of taxonomical schemes to include *Aspergillus nidulans* in this genus, despite the fact that its taxonomically correct name is *Emericella nidulans* (Samson, *In: Aspergillus*. Biology and Industrial Applications, pp 355-390, Ed. by Bennett and Klich, Boston)

[0004] EP417481 (Société des Produits Nestlé) thus describes a process for the production of a fermented soya sauce, in which a koji is prepared by mixing a koji culture with a mixture of cooked soya and roasted wheat, the koji is then hydrolyzed in aqueous suspension for 3 to 8 hours at 45°C to 60°C with the enzymes produced during fermentation of the koji culture, a moromi is further prepared by adding sodium chloride to the hydrolyzed koji suspension, the moromi is left to ferment and is then pressed and the liquor obtained is pasteurized and clarified.

[0005] EP429760 (Société des Produits Nestlé) describes a process for the production of a flavoring agent in which an aqueous suspension of a protein-rich material is prepared, the proteins are solubilized by hydrolysis of the suspension with a protease at pH6.0 to 11.0, the suspension is heat-treated at pH 4.6 to 6.5, and the suspension is ripened with enzymes of a koji culture.

[0006] Likewise, EP96201923.8 (Société des Produits Nestlé) describes a process for the production of a meat flavor, in which a mixture containing a vegetal proteinaceous source and a vegetal carbohydrates containing source is prepared, said mixture having initially at least 45% dry matter, the mixture is inoculated with a koji culture and by one or more another species of microorganisms involved in the traditional fermentation of meat, and the mixture is incubated until meat flavors are formed.

[0007] However, on the one hand, acid or alkaline hydrolysis can destroy the essential amino acids produced during hydrolysis thus reducing the nutritional value, whereas enzymatic hydrolysis rarely goes to completion so that the hydrolyzed protein contains substantial amounts of peptides. The optimization and further development of koji processes have been seriously hampered by the lack of knowledge on the nature of the hydrolytic enzymes, their regulation and how process parameters affect their expression and activity (e.g. temperature, pH, water activity, and salt concentration).

[0008] In the fungal *Emericella nidulans* (Katz *et al.*, Gene, 150, 287-292, 1994), fermentation activity is subject to at least three general control circuits including carbon catabolite repression, nitrogen and sulfur metabolite repression. These three regulatory circuits ensure that the available nitrogen-, carbon-, and sulfur sources in a substrate are utilized sequentially according to their nitrogen, energy and sulfur yield. Nitrogen metabolite repression is exerted by the *are*A gene product in *Emericella nidulans* (Arst *et al.*, Mol. Gen. Genet., 26, 111-141, 1973), whereas in the other fungals *Neurospora crassa* (Davies *et al.*, Proc. Natl. Acad. Sci. USA, 84, 3753-3757, 1987), *Penicillium chrysogenum* (Haas *et al.*, Curr. Genet., 27, 150-158, 1995) and *Saccharomyces cerevisiae* (Minehart *et al.*, Mol. Cell. Biol., 11, 6216-6228, 1991) similar genes exert a similar function.

[0009] The *are*A gene encodes a positively acting DNA-binding protein (AREA), belonging to the GATA family of transcription factors, that is required for the utilization of all nitrogen sources except ammonia or L-glutamine. Under nitrogen de-repressed conditions, signaled by high intracellular levels of glutamine, *are*A expression is down regulated by three mechanisms: 1) the AREA protein is inactivated, 2) *are*A transcription is halted and 3) by action of the 3' untranslated trailer sequence (3'-UTS) *are*A mRNA degradation is enhanced (Platt *et al.*, EMBO J., 15, 2791-2801, 1996). In the absence of a functional AREA protein, only ammonia or L-glutamine can be utilized as nitrogen source. Consequently, loss-of-function *are*A mutants can utilize only ammonia or L-glutamine as nitrogen sources (Arst *et al.*, 1973).

[0010] Observations in koji fermentation suggest that nitrogen metabolite repression is a major parameter in koji fermentation. For instance, high levels of L-glutamine are shown to negatively affect proteolytic activity in koji fermentation.

[0011] Furthermore, it has been observed that high levels of proteolytic activity and glutaminase activity are two mutually exclusive conditions in koji fermentation (Ushijima *et al.*, Agric. Biol. Chem., 51, 1051-1057, 1997). For instance, addition of 25mM L-glutamine into a minimal growth medium containing 0.1% wheat gluten reduces endoproteolytic enzyme activity about 40-50 fold. This phenomenon may be explained by postulating that L-glutamine is necessary for the induction of glutaminase. However, since L-glutamine is also the effector of nitrogen metabolite repression, the expression of proteolytic enzymes is suppressed when glutaminase is induced.

[0012] With regard to the fact that glutaminase suitably converts L-glutamine into L-glutamic acid which is an important natural taste enhancer (see WO95/31114), there is hence a need to overcome L-glutamine mediated suppression of proteolytic enzymes, allowing simultaneous expression of glutaminase and proteolytic enzymes in koji molds.

[0013] In addition, depending on the nature of the protein and the enzymes used for proteolysis, the peptides formed can however have extremely bitter tastes and are thus organoleptically undesirable. There is hence also a need for methods of hydrolyzing proteins leading to high degree of protein hydrolysis and to hydrolysates with excellent organoleptic properties.

[0014] Finally, biochemical analysis of residual peptides in cereals hydrolyzed by *koji* molds, e.g. wheat gluten, shows that a considerable amount of L-glutamine remains sequestered in proline containing peptides (Adler-Nissen, *In:* Enzymatic hydrolysis of food proteins. Elsevier Applied Sciences Publishers LTD, p120, 1986). There is hence also a need for methods of hydrolyzing proteins leading to liberation of high amount of L-glutamine.

Summary of the invention

[0015] The present invention has for object a koji mold which is capable to express at least 2 times more endo- and exo-peptidases than the wild type strain *Aspergillus oryzae* CNCM I-1882, and especially at least 30 mU of endopeptidase activity, at least 30 mU of leucine-amino-peptidase activity and at least 10 mU of prolyl-dipeptidyl-peptidase activity per ml of supernatant when grown in a minimal medium containing 0.2% soy bean proteins.

[0016] In a second aspect, the invention also provides a DNA-binding protein of *Aspergillus oryzae* (AREA) having at least the amino-acid sequence from amino-acid 1 to amino-acid 731 of SEQ ID NO:2 or functional derivatives thereof.

[0017] In a third aspect, the invention provides a DNA molecule that comprises an *are*A gene encoding the DNA-binding protein according to the invention.

[0018] In a fourth aspect, the invention provides a method for over-producing proteolytic enzymes, comprising cultivating a koji mold according to the invention in a suitable growth medium under conditions that the mold expresses enzymes, and optionally isolating the enzymes in the form of a concentrate.

[0019] In another aspect, the invention provides the use of the koji mold of the invention to hydrolyze protein-containing materials.

[0020] In a last further aspect, the invention provides a food product comprising a protein hydrolysate obtainable by fermentation with a koji mold of the invention of a material comprising proteins and at least 5mM of L-glutamine.

Detailed description of the invention

[0021] Within the following description, the percentages are given by weight except where otherwise stated. The amino acid or nucleotide sequences referred as "SEQ ID NO:" are always presented in the sequence listing hereafter. One leucine-aminopeptidase enzyme unit is defined as the amount of enzyme which produces 1 μmol *p*-nitroaniline per minute at 37°C from the substrate leucine-*p*-nitroanilide (absorption measured at 400nm; ε= 10'500 $M^{-1}cm^{-1}$). One prolyl-dipeptidyl-peptidase enzyme unit is defined as the amount of enzyme which produces 1 μmol *p*-nitroaniline per minute at 37°C from the substrate Alanine-Proline-*p*-nitroanilide (absorption measured at 400nm; ε= 10'500 $M^{-1}cm^{-1}$). One endopeptidase enzyme unit is defined as the amount of enzymes which produces 1 μmol of TCA-soluble peptides per minute at 37°C from the resorufin-labeled casein substrate under prescribed conditions (Boehringer Cat No. 1080733; absorption measured at 574nm).

[0022] The term "koji" is defined as the product of the fermentation with a koji mold culture of a mixture of a source of proteins and a source of carbohydrates, especially of a mixture of a leguminous plant or of a cooked oleaginous plant and of a cooked or roasted cereal source, for example of a mixture of soya or cooked beans and of cooked or roasted wheat or rice.

[0023] Likewise, the expression "functional derivative of an enzyme" includes all amino acid sequences which differ by substitution, deletion, addition of some amino acids, for instance 1-20 amino acids, but which keep their original activities or functions. The selection of a functional derivative is considered to be obvious to one skilled in the art, since one may easily creates variants of the truncated AREA protein (see SEQ ID NO:2) by slightly adapting methods known to one skilled in the art, for instance the methods described by Adams *et al.* (EP402450; Genencor), by Dunn *et al.* (Protein Engineering, 2, 283-291, 1988), by Greener *et al.* (Strategies, 7, 32-34, 1994), and/or by Deng *et al.* (Anal. Biochem, 200, 81, 1992).

[0024] In particular, a protein may be generally considered as a derivative to another protein, if its sequence is at least 85% identical to the protein, preferably at least 90%, in particular 99%. In the context of the present disclosure, the identity is determined by the ratio between the number of amino acids of a derivative sequence which are identical to those of the truncated AREA protein (see SEQ ID NO:2) and the total number of or amino acids of the said derivative sequence.

[0025] The present invention thus concerns any koji molds providing an enhanced expression of proteolytic enzymes, leading to high degree of protein hydrolysis and to hydrolysates with excellent organoleptic properties. Accordingly, these koji molds express (1) high levels of endopeptidases such as those capable to produce TCA-soluble peptides at 37°C from casein, and (2) high levels of exo-peptidases such as the leucine-amino-peptidase that eliminates N-terminal leucines (Deng *et al.*, Anal. Biochem., 200, 81, 1992) and the prolyl-dipeptidyl-peptidase which eliminates N-terminal X-Proline dipeptides, wherein X may be any amino-acid (Barrett *et al.*, In Mammalian Proteases: A Glossary and Bibliography, N.Y., Acad. Press, 2, p.132, 1986).

[0026] With regard to the fact that koji molds of the invention provide an enhanced prolyl-dipeptidyl-peptidase activity, they may suitably be used for liberating L-glutamine remains sequestered in proline containing peptides.

[0027] Koji molds providing the following enhanced expression of proteolytic enzymes are particularly adapted for the purpose of the invention: at least about 30 mU/ml*, preferably at least about 50 mU/ml* of endopeptidase activity; at least about 30 mU/ml*, preferably at least about 50 mU/ml* of leucine-amino-peptidase activity; and at least 10 mU/ml*, preferably at least about 15 mU/ml* of proline-dipeptidyl-peptidase activity (* per ml of supernatant when grown in a minimal medium containing 0.2% soy bean proteins).

[0028] In addition, koji molds that overcome L-glutamine mediated suppression of proteolytic enzymes, allowing simultaneous expression of glutaminase and proteolytic enzymes, are also part of the invention. These koji molds thus may express the above-mentioned proteolytic activities when grown in a minimal medium containing 0.2% soy bean proteins and at least 5 mM L-glutamine (0.073% w/w), for instance.

[0029] Koji molds of the invention may be obtained by random U.V and/or chemical mutagenesis, followed by selection of mutagenised koji mold providing the required phenotypic characteristics.

[0030] Selection of mutagenised koji mold particularly containing a mutagenised *are*A gene which is not repressed, when the mutagenised mold is grown in a minimal medium containing repressive amounts of L-glutamine, suitably achieved the needs of the present invention. To this end, *are*A mutants may be easily selected by classical random mutagenesis (UV, chemical) and selection on plates containing about 100 mM methyl ammonium chloride and 0.2% soy protein, for example.

[0031] It has to be noted that the prolyl-dipeptidyl-peptidase activity that is not naturally controlled by the *are*A gene expression, is enhanced against all expectations when the *are*A gene is de-repressed. Since expression of the prolyl-dipeptidyl-peptidase activity is induced by peptides (unpublished results), this AREA-dependent increase in activity may in fact be caused by the enhanced liberation of peptides by the endoproteases that are under *are*A control.

[0032] With regard to the fact that random U.V and/or chemical mutagenesis is time consuming, it would be also more adequate to construct koji molds of the invention by recombinant technology. Accordingly, a koji mold of the invention may preferably contain a recombinant *are*A gene which is truncated so as the C-terminally truncated AREA protein remains functional but not repressed when the mold is grown in a minimal medium containing repressive amounts of L-glutamine. It has to be noted that this truncation leads also to an *are*A mRNA that is less sensitive to mRNA degradation.

[0033] Truncation may be effected by cutting the native *are*A gene to a pre-determined region, and by introducing a terminater region thus allowing transcription of a truncated *areA* mRNA. Truncation is preferably effected downstream of the sequence encoding the DNA binding domain of AREA, that can be easily identified by 17 amino acid loop bound two pairs of cystein residues. More precisely, truncation may be effected downstream of the *are*A sequence encoding the conservative amino-acid structure cystein-2X-cystein-17X-cystein-2X-cystein, wherein X is any amino-acids and the numbers 2 and 17 refer to the number of amino-acids (Caddick *et al.*, Antonie van leeuwenhoek, 65, 169-177, 1994). This truncation may be particularly carried out in the 100 amino-acids following the *are*A sequence encoding the DNA binding domain.

[0034] Any functional fungal *are*A gene may be used in the context of the present invention, and in particular any functional *are*A gene capable of hybridizing under stringent conditions to the *are*A gene of *Aspergillus oryzae* having the nucleotide sequence from nucleotide 1189 to nucleotide 3846 of SEQ ID NO:1 or functional derivatives thereof due to the degeneracy of the genetic code.

[0035] A functional *are*A gene may be obtained in substantially purified form by using the method described within the following examples from any strain of *Aspergillus oryzae*. Alternatively, an *are*A gene may be (1) detected also from other genera or species of fungals by use of DNA probes derived from the nucleotide sequence SEQ ID NO:1 in a stringent hybridization assay, and (2) recovered by the well known Reverse-PCR method by use of suitable primers derived from SEQ ID NO:1 encompassing the *areA* gene. In a further aspect, an *are*A gene may also be *in-vitro* synthesized and then multiplied by using the polymerase chain reaction, for instance.

[0036] A suitable truncated *are*A gene thus may particularly consist of the nucleotide sequence defined by nucleotides 1189-1604 and 1704-3480 of SEQ ID NO:1 (SEQ ID NO: 1 contains an intron) or functional derivatives thereof due to the degeneracy of the genetic code, for example. This truncated gene thus encodes for the AREA DNA-binding protein of *Aspergillus oryzae* having the amino-acid sequence from amino-acid 1 to amino-acid 731 of SEQ ID NO:2, that is required for the utilization of all nitrogen sources except ammonia or L-glutamine.

[0037] This truncated *are*A gene then may be introduced in a vector, e.g. a replicative plasmid or an integrative circular or linearized non replicative plasmid, and may be operably linked to regulatory sequences that regulate a different gene in the said organism of origin or that regulate a different gene in a foreign organism (promoter and/or a terminator), for example. A regulatory sequence other than the native regulatory sequence will generally be selected for its high efficiency or desirable characteristic, such as, in case of a promoter inducibility or high expression capacity, for example.

[0038] If heterologous expression is preferred, meaning that the gene of the invention is expressed in another organism than the original host (strain, variety, species, genus, family, order, class or division) the regulatory sequences are preferably derived from an organism similar or equal to the expression host. For example, if the expression host is an *Aspergillus*, then the regulatory sequences will be derived from *Aspergillus*. The promoter suitable for constitutive expression, preferably in a fungal host, may be a promoter from the following genes: glycerolaldhehyde-3-phosphate dehydrogenase, phospho-glycerate kinase, triose phosphate isomerase and acetamidase, for example. Promoter suitable for inducible expression, preferably in a fungal host, may be a promoter from the following genes: endoxylanase IIA, glucoamylase A, cellobiosehydrolase, amylase, invertase, alcohol dehydrogenase and amyloglucosidase. The selection of a desirable regulatory sequence operably linked to a sequence of the invention and capable of directing the expression of the said nucleotide sequence is considered to be obvious to one skilled in the art.

[0039] The vector may also comprise a selection marker to discriminate host cells into which the recombinant DNA material has been introduced from cells that do not comprise the said recombinant material. Such marker genes are, for example in case fungal expression is preferred, the known *ga*-2, *pyr*G, *pyr*4, *pyr*A, *trp*C, *amd*S or *arg*B genes. The DNA molecule may also comprise at least one suitable replication origin. Suitable transformation methods and suitable expression vectors provided with a suitable transcription promoter, suitable transcription termination signals and suitable marker genes for selecting transformed cells are already known in the literature for many organisms including different *Aspergillus*, *Rhizopus* and *Mucor*. In the event fungal expression is required, the expression system described in EP278355 (Novartis) may be thus particularly adapted.

[0040] Recombinant koji molds may be obtained by any method enabling a foreign DNA to be introduced into a cell. Such methods include transformation, electroporation, or any other technique known to those skilled in the art.

[0041] In the context of the present invention, koji molds are those traditionally used for making a koji culture including cells of the genus *Aspergillus* (ICBN taxonomy), *Rhizopus* and/or *Mucor*. Among those, the following species may be used, including *Aspergillus soyae*, *Aspergillus oryzae* (ATCC 20386), *Aspergillus phoenicis* (ATCC 14332), *Aspergillus niger* (ATCC 1004), *Aspergillus awamori* (ATCC 14331), *Rhizopus oryzae* (ATCC 4858), *Rhizopus oligosporus* (ATCC 22959), *Rhizopus japonicus* (ATCC 8466), *Rhizopus formosaensis*, *Mucor circinelloides* (ATCC 15242), *Mucor japanicus*, *Penicillium glaucum* and *Penicillium fuscum* (ATCC 10447). Strains referred by an ATCC number are accessible at the American Type Culture Collection, Rockville, Maryland 20852, US. The invention is not limited by such indications that were rather give to enable one skilled in the art to carry out the invention.

[0042] Recombinant cells of the invention may comprise the truncated *are*A gene stably integrated into the chromosome or on a replicative plasmid. Among all recombinant cells of the invention thus created, the present invention has particularly for object the strains *A. oryzae* CNCM I-1881, CNCM I-1883 and CNCM I-1884.

[0043] Preferably, only one functional truncated *are*A gene is integrated into the chromosome under the control of regulatory sequences that are native to the host organism.

[0044] In order to stably integrate into the chromosome of eucaryotic cells only one functional truncated *are*A gene which is fused to a promoter and a terminator which are native to the host organism, the DNA molecule of the invention may be integrated by slightly adapting the process of Ruiter-Jacobs *et al.* (Curr. Genet., 16, 159-163, 1989), i.e.,

[0045] (1) preparing a non-replicative DNA fragment by ligating the truncated *are*A gene, which is operably linked to a promoter and terminator that are native to the host organism, downstream the DNA sequence encoding an essential gene, said gene being inactivated by at least one mutation and/or one deletion (this essential gene may be any genes involved in RNA synthesis, such as the *pyr*G gene in case *A. oryzae* is used, for example); (2) selecting a host organism containing the essential gene which is however inactivated by another mutation(s) or deletion(s); (3) transforming said host organism with the non replicative DNA fragment; (4) identifying integrate transformants in which the DNA fragment is integrated so as to restore the native function of the essential gene; (5) selecting an integrate transformant in which only one DNA fragment is integrated.

[0046] Over-expression of the AREA DNA-binding protein may be obtained by incorporation of the truncated *are*A gene in an expression host, said *are*A gene being operably linked to one or more regulatory sequences which serve to increase expression levels of the AREA protein of the invention.

[0047] The over-expression can be further achieved by introducing (replicative plasmid) or integrating (by integration

in the genome) multiple copies of the functional truncated *are*A gene of the invention. As examples of koji molds containing multiple copies of a functional truncated *are*A genes, the transformants *Aspergilus oryzae* A (see example 1), *Aspergilus oryzae xpr*D1 (see example 3) and *Aspergilus oryzae* NF1 containing pNFF68 (see example 4) were deposited under the Budapest Treaty where they respectively receive the deposit numbers CNCM I-1881, CNCM I-1883 and CNCM I-1884.

[0048] The invention is also directed to a process for over-producing proteolytic enzymes comprising, providing koji mold of the invention in a suitable growth medium under conditions that the mold expresses proteolytic enzymes, and optionally isolating the enzymes in the form of a concentrate, for example by removing solids from the fermentation broth by centrifugation or filtration. The selection of the appropriate medium may be based on the choice of expression host and/or based on the regulatory requirements of the DNA recombinant material. Such media are well-known to those skilled in the art. After fermentation, the molds can be removed from the fermentation broth by centrifugation or filtration.

[0049] Typical L-glutamine concentrations reached during koji hydrolysis in liquid system may be 0.5-1% w/w, for example. The present koji molds are thus particularly adapted for hydrolyzing any protein containing materials, in particular those containing high amounts of L-glutamine (more than 5mM). These protein containing materials may be mixtures of a source of proteins and a source of carbohydrates, especially a mixture of a leguminous plant or of a cooked oleaginous plant and of a cooked or roasted cereal source, for example a mixture of soya or cooked beans and of cooked or roasted wheat or rice.

[0050] Compositions containing wheat gluten are particularly adapted for the purpose of the present invention, since high amounts of L-glutamine remains sequestered in proline containing peptides when wheat gluten is hydrolyzed by traditional koji cultures.

[0051] In the event one may try, after or during hydrolysis with koji molds, to further liberate as much as possible L-glutamine linked to proline residues, the present invention provides a method in which the koji mold of the invention of the invention is used in combination with at least an enzyme or a microorganism providing a prolidase activity, that is to say an enzyme which has a high level of specificity towards dipeptides of the X-Pro type (Ezespla *et al.*, Ap. Env. Microb., <u>63</u>, 314-316, 1997; Such kind of enzyme is already available from Sigma: E.C. 3.4.13.9).

[0052] In addition, the koji molds of the invention are particularly adapted for hydrolyzing protein containing materials that comprise at least 5mM of L-glutamine, allowing formation of L-glutamic acid which is an important natural taste enhancer and high degree of protein hydrolysates with excellent organoleptic properties.

[0053] In a further aspect, the present invention relates to food product comprising a protein hydrolysate obtainable by fermentation of a material comprising proteins and at least 5 mM of L-glutamine with a koji mold of the invention. Such food contains naturally high amounts of L-glutamic acid (and/or L-glutamate) and high degree of protein hydrolysates with excellent organoleptic properties leading to a non-bitter flavor and a significantly lower allergenicity than unhydrolyzed proteins

[0054] Important food product of the present invention is an ingredient of a mother milk substitute for infants, or a hydrolyzed vegetable protein ingredient. The milk substitute may be further formulated in substantially the same way as that indicated in the prior literature for products of this type (cf. EP 96202475.8).

[0055] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties to the extent necessary for understanding the present invention. DNA manipulation, cloning and transformation of bacteria cells are, except where otherwise stated, carried out according to the textbook of Sambrook *et al.* (Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, U.S.A., 1989). These examples are preceded by a brief description of the figures, of the plasmids and strains used, and by the composition of various media. The strains *A. oryzae* TK3, *Aspergilus oryzae* A (see example 1), *Aspergilus oryzae* NF2 (see example 2), *Aspergilus oryzae xpr*D1 (see example 3) and *Aspergilus oryzae* NF1 containing pNFF68 (example 4) were deposited under the Budapest Treaty, at the Collection Nationale de Culture de Microorganismes (CNCM), 25 rue du docteur Roux, 75724 Paris, France, on June 24, 1997, where they receive respectively the deposit numbers CNCM I-1882, CNCM I-1881, CNCM I-1885, CNCM I-1884, CNCM I-1883. All restrictions as to the availability of these deposits will be withdrawn upon first publication of this application or another application which claims benefit of priority to this application.

<u>Figures</u>

[0056]

- Figure 1 shows the restriction map of pNFF21 which comprises the truncated *E. nidulans are*A gene and the *pki*A

promotor and terminater.

- Figure 2 shows the relative Endo, LAP and DPPIV activities of *A. oryzae* TK3 (wild type), *A. oryzae* transformed by pNFF28 encompassing the *pyr*G gene (control *pyr*+), *A. oryzae are*A disruption mutant (control *are*A-; see example 2), and 3 mutants of *A. oryzae* NF1 which were cotransformed with pNFF28 and pNFF21.

- Figure 3 shows the restriction map of the 4.6 kb *Eco*RI-*Hind*III insert of plasmid pNFF5, which complements the *are*A19 mutation in *Emericella nidulans* G332; both exons encompassing the coding region are indicated with solid arrows.

- Figure 4 shows the *are*A disruption construct pNFF44 containing the two exons of the *E. nidulans pyr*G gene (*pyr*1 and *pyr*2), the two exons of *A. oryzae are*A gene (*are*A1 and *are*A2) and the bacterial kanamycin resistance gene (*Kana*R).

- Figure 5 shows the site directed mutagenesis of the *A. oryzae are*A gene; the mismatches in the mutagenic primer with the wild type *are*A sequence are indicted as follows: the stop codon (TAA) is italic, the *Afl*II site doubly underlined and the introduced *Eco*RV site is marked in bold print and is underlined.

- Figure 6 shows the relative Endo, LAP and DPPIV activities of *A. oryzae* TK3 (wild type) and 9 mutants of *A. oryzae* NF1 which were co-transformed with de-repressed *are*A amplification product and the *pyr*G amplification product. and transformants were selected on MM with glucose and glutamine.

Strains & plasmids

[0057]

- *E. nidulans* G191 (*pyr*G89, *fwn*A1, *paba*A1, *Yu*A1), *E. nidulans* G353 (*are*A1, *bi*A1) and *E. nidulans* G332 (*paba*A1, *y*A2, *xpr*D1) were obtained from the Glasgow Genetic Stock Center via Dr. A.J. Clutterbuck. Other wild type strains of Emericella nidulans also may have been used in the following examples.
- *Aspergillus oryzae* TK3 was obtained from the strain collection of Nestlé.
- *Aspergillus oryzae* NF1 (*pyr*G1) is a uridine auxotroph derivative of *A. oryzae* TK3 in which the *pyr*G gene, encoding orotidine 5'-phosphate decarboxylase, was inactivated by targeted disruption.
- *Escherichia coli* BZ 234 (Collection from the Biozenter, University of Basel, Basel, Switzerland) was used as host for the propagation of plasmids. *E. coli* strains JM109 (*end*A1, *rec*A1, *gyr*A96, *hsd*R17 (r_K-, m_{K+}), *rel*A1, *sup*E44, λ^-, Δ(*lac-pro*AB), [F', *tra*D36, *pro*A^+B^+, *lac*$I^qZ\Delta M15$]) and EM1301 (*lac*Z53, *mut*S201::Tn5, *thy*A36, *rha*-5, *met*B1, *deo*C, IN(*rrn*D-*rrn*E)) were used in the site directed mutagenesis.

- The plasmid pHELP1 was used for direct cloning in *Emericella nidulans* (Gems and Clutterbuck, Curr. Genet., 24, 520-524, 1993; GenBank accession number: X78051).
- Plasmid pNFF28 contains the *A. oryzae* TK3 *pyr*G gene (GenBank accession number: Y13811).
- Plasmid pFBY182, containing the *pep*B gene as a *Eco*RI-*Xba*I fragment under the control of the *Aspergillus niger pki*A promoter and terminator was obtained from Novartis, Switzerland, via Dr. Gabor Jarai (GenBank accession number: S38698).
- pNEB193 (New England Biolabs), pAlter1 (Promega), pBluescriptSK$^-$ (Stratagene), pHSS19 and pGEM-T (Promega), and pK18 (GenBank accession number: M17626) were used for subcloning.

Media

[0058] Fungal Nitrogen Base (FNB) was composed of 1x Yeast Nitrogen Base (YNB) without amino acids and $(NH_4)_2SO_4$ (Difco) with 50 mM glucose as carbon source and 10 mM $NaNO_3$ as nitrogen source. In the case of *E. nidulans* G353 (*are*A1, *bi*A1), 10 mM glutamine was added as nitrogen source. Growth tests were performed on MM (which contains per litre 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, Pontecorvo, 1953) only now 10 mM $NaNO_3$ served as sole nitrogen source. Protease plate assays were performed on MM with 0.2% soy protein as sole carbon and nitrogen source. For quantitative studies 250 ml conical flasks filled with 80 ml of MM with 0.2% soy protein, as sole nitrogen and carbon source, were inoculated with 10^6 conidiospores/ml and incubated for 5 days at 30° C without agitation.

Exemple 1 Over-expression of the *E. nidulans* truncated *are*A gene

[0059] To assess the feasibility of increasing expression of proteolytic enzymes by modulation of *are*A expression, we decided to overexpress the *Emericella nidulans* gene in *A. oryzae* TK3.

[0060] To this end, amplification of the coding region of the *are*A gene from *Emericella nidulans* G191 and cloning of the PCR product into the expression vector pFBY182 were achieved as follows: with oligonucleotides SEQ ID NO:3 and SEQ ID NO:4, a 2.174 bp fragment, encompassing the *are*A coding region between positions 2009 and 4168, was amplified from genomic DNA of *E. nidulans* G191. At the same time an *Eco*RI site was added to 5' end and a *Xba*I site to the 3' end, allowing directional cloning into *Eco*RI-*Xba*I digested fungal expression vector pFBY182 to give pNFF21 (see figure 1). In pNFF21, *are*A transcription is under control of the *A. niger pki*A promoter and terminator (Graaff, Curr. Genet., 22, 21-27, 1992), thereby preventing the down-regulation under repressing conditions exerted by its native 3' UTS.

[0061] pNFF21 was introduced into *A. oryzae* NF1 (*pyr*G1) by co-transformation with pNFF28 containing the *A. oryzae pyr*G gene. Accordingly, *A. oryzae* NF1 was grown in MM with 0.1% yeast extract (Difco), 50 mM glucose and 5 mM glutamine. The mycelium was harvested by sterile filtration, washed once with sterile double distilled water and once with K0.8MC (20 mM MES-HCl pH 5.8, 0.8 M KCl, 50 mM $CaCl_2$). 1.5 g of mycelium was resuspended in 20 ml of a filter sterilized 5 mg/ml solution of Novozyme 234 in K0.8MC. The mycelium suspension was incubated at 30°C for 2 hours with gentle agitation (120 rpm). The protoplasts were liberated from the mycelium by gentle resuspension with a pipet, washed twice with 20 ml of S1.0TC (10 mM Tris-HCl pH 7.5, 1 M Sorbitol, 50 mM $CaCl_2$) and were resuspended in a final concentration of 10^8/ml in S1.0TC. 20 ml of DNA was mixed with 200 µl of protoplasts and 50 µl of 25% PEG 6000 in 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$ and incubated for 20 min on ice. To this mixture, 2 ml of 25% PEG 6000 (BDH) in 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$ were added, gently mixed and incubated for 5 min at room temperature. 4 ml of S1.0TC was added and 1.0 ml aliquots were mixed with 5 ml of 2% low melting point agarose (Sigma) in OFNB (osmotically stabilized fungal nitrogen base) and plated onto OFNB agar (Difco) with 50 mM glucose and 10 mM $NaNO_3$. *A. oryzae* NF1 transformants were plated on MM agar with 1 M sucrose, 50 mM glucose and 5 mM glutamine.

[0062] The resulting transformants were screened on MM containing 2% soy protein. Among 20 transformants screened, three showed increased secretion of proteolytic activity as judged from the sizes of the halo surrounding the colony after 36 hours of incubation at 30°C (transformants A, B and C). These three transformants were grown for five days at 30°C in stationary liquid cultures in MM with 0.2% soy protein and analyzed for proteolytic activity with the appropriate controls.

[0063] To this end, conidiospores (10^6/ml) of these three strains were used to inoculate 80 ml of liquid MM with 0.2% soy protein as sole nitrogen and carbon source. These cultures were incubated for 5 days at 30°C without agitation. After filtration to remove the mycelium, the medium was assayed for endoproteolytic activity (Endo), Leucine aminopeptidase activity (Lap) and proline-dipeptidyl-peptidase activity (DPPIV). Endoproteolytic enzyme activity was measured with resorufin-labeled casein according to Boehringer method description supplied with the substrate (Resorufin-labeled casein, Cat.No. 1080733). Leucine aminopeptidase and dipeptidyl peptidase IV activities were determined by UV spectrometry with synthetic substrates Leu-pNa and Ala-Pro-pNa (Bachem, Switzerland), respectively, according to Sarath *et al.* (*In* Protease assay methods for proteolytic enzymes: a practical approach, Beynon R.J., Bond J.S., eds., IRL Press, Oxford). 10 mM substrate stock solution in dimethylsulfoxide (DMSO) was diluted with 100 mM sodium phosphate buffer, pH 7.0, to a final concentration of 0.5 mM. 20-100 µl culture medium supernatant was added and reaction proceeded for up to 60 min at 37°C. A control with blank substrate and blank supernatant was assayed in parallel. The release of the chromophoric group 4-nitroaniline (ε: 10'500 $M^{-1}cm^{-1}$) was measured at 400 nm and activities were expressed as mU/ml (nmol/min/ml).

[0064] Relative proteolytic activities are shown in figure 2. In the *are*A disruption mutant endoproteolytic (Endo) and leucine aminopeptidase (Lap) activity are significantly reduced compared to TK3 and the *pyr+* control strains, whereas proline dipeptidyl peptidase activity (DPPIV) is not affected. Apparently, proline dipeptidylpeptidase expression is not under *are*A control. Introduction of multiple copies of *E. nidulans are*A in *A. oryzae* NF1 under the control of the *pki*A expression signals results in over-expression of endoproteolytic, leucine aminopeptidase and proline-dipeptidyl-peptidase enzyme activity.

Example 2 Over-expression of the *A. oryzae* truncated *are*A gene

[0065]

1) Cloning of the *A. oryzae are*A gene: the *A. oryzae are*A gene was cloned by complementation of the corresponding *are*A gene of *E. nidulans* with the instant library method (Gems *et al.*, 1993).

First of all, the isolation of the genomic DNA was performed according to a modified protocol of the method described by Raeder and Broda (Let. appl. Microbiol., 1, 17-20, 1985). Mycelium was harvested by filtration, imme-

diately frozen in liquid nitrogen and lyophilized. It was then reduced to a fine powder using a mortar and pestle. 200 mg of the powdered mycelium was resuspended in 2.5 ml of extraction buffer (200 mM Tris-HCl pH 8.5 150 mM NaCl, 25 mM EDTA, 0.5 % SDS) and the solution was extracted with 1.75 ml extraction buffer-equilibrated phenol and 0.75 ml of chloroform/isoamylalcohol (24:1, v/v). The mixture was centrifuged (20 min, 3000 g). The aqueous phase was retrieved and incubated with 125 µl of RNAse A (Boehringer) solution (10 mg/ml) for 10 min at 37°C. 1.25 ml of 2-propanol (Merck) were then added. The pellet was washed with 70 % ethanol and finally resuspended in 500 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). 500 ml of 2 x QBT (1.5 M NaCl, 100 mM MOPS, 30 % ethanol, pH 7.0) were added to the sample which was then applied to a "Genomic-tip 100" (Qiagen), rinsed and eluted as recommended by the supplier.

Cloning by complementation was then achieved by mixing 40 µg *Bam*HI digested pHELP1 with either 100 µg *Bam*HI digested or 100 µg partially *Sau*3A digested genomic DNA from *A. oryzae* TK3. Additionally, 40 µg *Kpn*I digested pHELP1 was mixed with 100 µg *Kpn*I digested genomic DNA from *A. oryzae* TK3. All tree DNA mixes were introduced into *E. nidulans* G332 and transformants were selected on osmotically stabilized FNB medium with $NaNO_3$ as sole nitrogen source.

The transformation experiment with the partially digested *Sau*3A *A. oryzae* TK3 DNA, did not yield any transformants. By contrast the experiments with the *Bam*HI and *Kpn*I digested *A. oryzae* TK3 DNA did yield 14 and 3 transformants respectively. Again these transformants exhibited irregular growth, which suggested that the complementing gene was located on an autonomously replicating plasmid. In a separate experiment 40 µg *Kpn*I digested pHELP1 was co-transformed with 100 µg *Kpn*I digested genomic DNA from *E. nidulans* G332 (*xpr*D1) and one transformant was obtained.

From three *Bam*HI derived transformants and one *Kpn*I derived *are*A transformant, plasmids were rescued by transformation of *E. coli*. No plasmids could be isolated from the transformant from the *xpr*D1 transformation. From each individual *E. nidulans Bam*HI *are*A$^+$ transformant several plasmids could be recovered. Restriction analysis of these plasmids showed that they were pHELP1 derivatives containing additional restriction fragments, but that not all of these inserts carried terminal *Bam*HI sites. Similarly, from the *Kpn*I *are*A$^+$ transformant several pHELP1 derivatives could be recovered, non of which had an insert with terminal *Kpn*I sites. These observations indicate instability of the plasmids

One *Bam*HI (pNFF3) and one *Kpn*I (pNFF4) pHELP1 derivative were chosen for further analysis. The inserts of both clones hybridized to the coding region of the *E. nidulans are*A gene. Detailed analysis of these two clones showed that in pNFF3, the entire *are*A gene was located on a 4.6 kb *Eco*RI-*Hind*III fragment (Fig. 3). This 4.6 kb *Eco*RI-*Hind*III fragment was subcloned into pHSS19 to give pNFF5. Upon re-introduction into *E. nidulans* G323, pNFF5 restores its ability to grow on $NaNO_3$ as sole nitrogen source demonstrating that this plasmid contains a functional *are*A gene (data not shown).

<u>2) Characterization of the *A. oryzae are*A gene</u>: the complete nucleotide sequence of the *Eco*RI-*Hind*III insert of pNFF5 was determined by analysis of both strands on partially overlapping subclones. The nucleotide sequence was determined, on a Licor model 4000 automatic sequencer. IRD41 labeled primers were used for sequencing both strands of partially overlapping subclones by the dideoxynucleotide method of Sanger *et al.* (Proc Natl Acad Sci USA, <u>74</u>, 5463-5467, 1977). The DNA sequence analysis was performed by using the GCG Computer programs (Devereux *et al.*, Nucl. Acids Res., <u>12</u>, 387-395, 1987).

Results show that the *A. oryzae are*A gene encodes a protein of 853 amino acid residues with a deduced molecular weight of 91.5 kDa (see SEQ ID NO:2). At the protein level the *A. oryzae are*A exhibits a similarity of 83% and at the DNA level 70% similarity with the *E. nidulans are*A gene.

Moreover, in the putative promoter region the overall DNA homology with *E. nidulans* drops to 43%. Still, seven stretches of DNA 5 to 13 bp long show 100% sequence identity and occupy virtually identical positions in both promoters. These sequences could represent *cis*-acting elements. Additionally, the 5' non-transcribed region contains several putative AREA-binding sites (GATA or TATC; Fu and Marzluf, Proc. Natl. Acad. Sci USA, <u>87</u>, 5351-5355, 1990) two of which occupy identical positions as the two functional AREA-binding sites in *E. nidulans*.

<u>3) Disruption of the *A. oryzae are*A gene:</u> to elucidate the role of *are*A in the expression of protease encoding genes, an *are*A-null mutant was generated by gene disruption. To construct such an *are*A null allele, the two internal *Sma*I fragments (see Fig. 3) were removed from pNFF5 to give pNFF10. To do so, pNFF10 was created by digesting pNFF5, containing the *A. oryzae* TK3 *are*A gene, with *Sma*I and selfligating the vector containing fragment. This deleted the internal 0.5 and 0.2 kb *Sma*I fragments from the second exon of the *are*A gene in pNFF5

As selection marker, a PCR product, encompassing the *E. nidulans pyr*G gene, was inserted into the unique *Sma*I site of pNFF10 to give pNFF44 (Fig.4). Accordingly, with oligonucleotides SEQ ID NO:5 and SEQ ID NO: 6 the *pyr*G gene was amplified from *E. nidulans* G332 and the 1.851 bp PCR product cloned into pGEM-T (Promega) to give pNFF38 and pNFF39. The *Eco*RI fragment, encompassing the *pyr*G gene was retrieved from pNFF39, blunt ended with T4 DNA polymerase and cloned into the *Sma*I site of pNFF10.

This pNFF44 construct, linearized with *Eco*RI and *Nhe*I, was used to transform *A. oryzae* NF1, and transform-

ants were selected on osmotically stabilized MM containing glucose and glutamine as carbon and nitrogen source respectively. All *pyr*G$^+$ transformants were further checked for their ability to use nitrate and soy protein as sole nitrogen sources. Four *pyr*G$^+$ transformants exhibited greatly reduced or no growth on nitrate MM and three did not form a halo when grown for two days on MM containing 0.2% soy protein as sole nitrogen and carbon source (data not shown). A Southern blot of *Sma*I digested genomic DNA of these four and six other *pyr*G$^+$ transformants was digested with *Sma*I and probed with the 4.6 kb *Eco*RI-*Hind*III insert of pNFF5. Only in one of the transformants the two internal *Sma*I fragments of the *are*A gene were deleted, identifying this transformant as an *are*A null-mutant. This *are*A disruption mutant was called NF2.

The *are*A mutant NF2 was grown for 5 days at 30°C without agitation in 80 ml of MM with 0.2% soy protein. The *are*A mutant grew poorly on MM with 0.2% soy protein. Analysis of the culture broth showed a 75% decrease in total endoproteolytic activity and a 60% decrease in leucine aminopeptidase activity compared to the *A. oryzae* TK3 (WT) control (Fig 2). By contrast the proline dipeptidylpeptidase activity in the *are*A mutant did not significantly differ from the wild type control (Fig. 2).

4) Construction of a constitutive *are*A allele : co-transformation experiments with pNFF5, containing the WT *are*A gene, did not yield co-transformants that overproduced proteolytic enzymes (data not shown). This suggested tight regulation of the *A. oryzae* *are*A gene.

[0066] To allow the constitutive expression of proteolytic enzymes (i.e. in the presence of glutamine), truncation of the *are*A gene was achieved. By site directed mutagenesis, a stop codon (TAA), an *Afl*II and an *Eco*RV site were introduced into the 4.6 kb *Eco*RI-*Hind*III *are*A fragment, truncating the AREA protein after amino acid residue 752 (see figure 5).

[0067] To this end, the *Eco*RI-*Hind*III insert of pNFF5 was ligated into pALTER1 and introduced into *E. coli* JM109 to give pNFF49. By superinfection with the helperphage M13KO7, single stranded DNA was generated from pNFF49 which was used in the site directed mutagenesis procedure with the Altered sites II kit (Promega). Then 0.05 pmol single stranded pNFF49 was annealed to 0.25 pmol Ampicillin repair oligonucleotide SEQ ID NO:7, 0.25 pmol Tetracycline knockout oligonucleotide SEQ ID NO: 8 and 1.25 pmol *are*A/*xpr*D1 mutagenic oligonucleotide SEQ ID NO:9, in 20 ml of 20 mM Tris-HCl pH 7.5,10 mM $MgCl_2$ and 50 mM NaCl in a Perkin Elmer Thermocycler programmed to heat the annealing mixture to 75°C for 5 min and then to cool to 45° C at a rate of 1°C/min. From 45°C to 20° the cooling rate was increased to 1.5°C/min. Next 3 ml 100 mM Tris-HCl pH 7.5, 5 mM dNTPs, 10 mM ATP and 20 mM DTT were added. The mixture was incubated for 90 min at 37°C with 5U T4 DNA polymerase and 1U T4 DNA ligase. A 3 ml aliquot of the reaction mixture was introduced into *E. coli* ES1301 by electroporation and transformants were selected in 5 ml LB containing 125 mg/ml ampicillin. The mutagenised plasmids were recovered from ES1301 and introduced into BZ234.

[0068] The 3.5 kb *Eco*RI-*Eco*RV fragment was further cloned into pBlueskript to give pNFF58. To test functionality pNFF58 was introduced into *A. oryzae* NF2 (see above) and transformants were selected on OFNB containing $NaNO_3$ as sole nitrogen source. With pNFF58, 1.5 transformants/μg were obtained and with the control pNFF5, 6 transformants/μg. These data prove that pNFF58 still contains a functional *are*A gene. The pNFF58 transformants were screened for proteolytic activity on MM with 0.2% soy protein and MM with 0.2% soy protein and 10 mM glutamine. On 0.2% soy protein several transformants produced bigger halos that the wild type control (*A. oryzae* TK3) suggesting that overexpression results in enhanced secretion of proteolytic enzymes. Most transformants produced halos on both media, suggesting derepressed expression of proteolytic enzymes (data not shown).

Example 3 Construction of protease-overproducing Koji mould strains.

[0069] In order to produce potential production koji mold strains, at least one additional copy of the de-repressed *are*A allele would need to be introduced into the *A. oryzae* TK3 derivative NF1. For legal reasons, this had to be done without introducing bacterial sequences, especially antibiotic resistance genes. To this end the inserts of pNFF28 and pNFF58 were amplified by PCR with *Pfu*I DNA polymerase and phosphorylated oligonucleotides SEQ ID NO:10 and SEQ ID NO:11. The amplification products were selfligated and purified. 10 μg of the pNFF58 amplification product and 10 μg of the pNFF28 amplification product were introduced into *A. oryzae* NF1 and the transformants were selected on osmotically stabilised MM with 50 mM glucose and 5 mM glutamine. As a control also 10 μg of pNFF28 was introduced. The plasmid pNFF28 yielded 30 transformants/μg, the pNFF28 PCR product 6 transformants/μg and the pNFF28/pNFF58 PCR products 16 transformants/μg.

[0070] The potential co-transformants were screened for increased protease activity on MM with 0.2% soy protein and MM with 0.2% soy protein and 10 mM L-glutamine. Twelve transformants produced more proteolytic activity on both media as indicated by the increased size of the halo they produced. To quantify the overexpression, nine of them were incubated without agitation for 5 days at 30°C in 80 ml MM containing 0.2% soy protein. The culture media were assayed for proteolytic activity (Fig. 6).

[0071] As with the *E. nidulans* *are*A gene under control of the *A. niger* *pki*A expression signals (Fig. 2) all three

classes of proteolytic activity tested were increased compared to the *A. oryzae* TK3 wild type and a *pyr*G+ derivative of *A. oryzae* NF1.

[0072] Southern analysis of the protease overproducing strains showed that all co-transformants contain 2 to 4 functionally integrated copies of the de-repressed *areA* gene.

Comparing the observed levels of protease overproduction and the number of functionally integrated copies of de-repressed *are*A gene, no clear relation was observed. Transformant *xpr*D1 produces the highest level of proteolytic activity and contains multiple copies of functionally integrated *xpr*D1. However, transformant *xpr*D12 contains far less copies of functionally integrated *xpr*D1 but produces almost as much activity as transformant *xpr*D1. Furthermore, the hybridisation patterns of *xpr*D6 and *xpr*D7 are very similar, yet *xpr*D6 overproduces all activities tested 1.5 fold but *xpr*D7 overproduces only proline dipeptidylpeptidase.

Example 4 Expression of *A. oryzae xpr*D1 allele with the promoter and terminater of the *A. oryzae dpp*IV gene

[0073] Co-transformation experiments of example 2 resulted in strains that had muliple copies of pNFF58 integrated in the genome and that overproduced proteolytic activity 2 to 3 fold when compare to the wild type TK3 strain. By contrast, strains with one copy of pNFF21 (example 1), where *E. nidulans are*A is under the control of a strong glycolytic promoter resulted in 6 fold over-expression. These data suggest that the native *are*A promoter is a weak promoter and that expression of a functional truncated *are*A under control of a strong promoter gives better results.

[0074] To this end, the *dpp*IV gene of *A. oryzae* TK3 was amplified by PCR with *Pfu*I DNA polymerase and phosphorylated oligonucleotides SEQ ID NO:12 and SEQ ID NO:13. The PCR product was then digested with *Apa*I and *Eco*RV enzymes. The digested *Apa*I-*Eco*RV 4.8 kb fragment was subcloned into pALTER1 (Promega) to give pNFF61. Next pNFF61 was subjected to a site directed mutagenesis according to the protocol of Deng *et al.* (Anal. Biochem., 200, 81, 1992), using the 5'-phosphorylated mutagenic oligonucleotides SEQ ID NO:14 and SEQ ID NO:15 according to the manual with Altered sites II kit (Promega) resulting in pNFF62. Using the polymerase enzyme *Pfu*I and the oligonuclotides SEQ ID NO:16 and SEQ ID NO:17, the *xpr*D1 allele was amplified by PCR, from pNFF58 containing the *A. oryzae xpr*D1 allele, as a 3.4 kb *Eco*RI-*Eco*RV fragment. The 2294 bp *xpr*D1 amplification product was then phosphorylated and cloned into the *Sma*I digested vector pK19 (Pridmore *et al.*, Gene, 56, 309-312, 1987) to give pNFF64. Finally the *Not*I-*Ecl*136II insert from pNFF64 was inserted into *Not*I-*Hpa*I pNFF62 creating pNFF68 encompassing the *xpr*D1 allele fused to the *dpp*IV promoter and terminater.

[0075] PNFF68 was intoduced into *A. oryzae* NF1 by co-transformation with pNFF28, and primary transformants were screened for increased proteolytic activity on MM plates containing 0.2% soy protein. Five out of 35 transformants exhibited increased halo sizes compared to *A. oryzae* TK3. Among the 5 transformants thus selected, one was deposited under the Budapest Treaty at the CNCM, where it receives the deposit number CNCM I-1883.

[0076] Co-transformants over-expressing proteolytic enzymes and wild type controls were plated on MM plates containing 0.2% soy protein and 5 mM L-glutamine. All the selected co-transformants still produced a halo in the presence of 5 mM glutamine, whereas the wild type did not, indicating de-repressed expression of proteolytic activity.

[0077] To quantify the over-expression, transformants were incubated without agitation for 5 days at 30°C in 80 ml MM containing 0.2% soy protein. The culture media were then assayed for proteolytic activity. Results show an overproduction of proteolytic activity of at least 6 fold when compare to the wild type TK3 strain.

Examples 5

[0078] For preparing a fermented soya sauce, a koji is prepared by mixing an *Aspergillus oryzae* CNCM I-1883 koji culture with a mixture of cooked soya and roasted wheat, the koji is then hydrolyzed in aqueous suspension for 3 to 8 hours at 45°C to 60°C with the enzymes produced during fermentation of the *Aspergillus oryzae* CNCM I-1 culture, a moromi is further prepared by adding suitable amount of sodium chloride to the hydrolyzed koji suspension, the moromi is left to ferment and is then pressed and the liquor obtained is pasteurized and clarified.

Examples 6

[0079] For producing a flavouring agent, a aqueous suspension of a mixture of cooked soya and roasted wheat is prepared, the proteins are solubilized by hydrolysis of the suspension with a protease at pH6.0 to 11.0, the suspension is heat-trated at pH 4.6 to 6.5, and the suspension is ripened with the prolidase enzyme of Sigma and proteolytic enzymes which have been isolated from a liquid medium fermented by *Aspergillus oryzae* CNCM I-1881.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(ii) (A) NAME: SOCIETE DES PRODUITS NESTLE
(B) STREET: AVENUE NESTLE 55
(C) CITY: VEVEY
(D) STATE: VAUD
(E) COUNTRY: SWITZERLAND
(F) POSTAL CODE (ZIP): 1500
(ii) TITLE OF INVENTION: ENHANCED EXPRESSION OF PROTEOLYTIC ENZYMES IN KOJI MOLDS
(iii) NUMBER OF SEQUENCES: 17
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4657 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1189..1604
(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:1605..1703
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1704..3846
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1189..3480
(D) OTHER INFORMATION:/label= TRUNCATED-AREA
/note = "AREA IS TRUNCATED IMMEDIATELY DOWNSTREAM THE SEQUENCE ENCODING A DNA BINDING DOMAIN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GGTTGGGAGA ACAGTGCCTT CAAACAAGCC TTCATACCAT GCTACTTGAC TAGTCAGGGA   180
CTAGTCACCA AGTAATCTAG ATAGGACTTG CCTTTGGCCT CCATCAGTTC CTTCATAGTG   240
GGAGGTCCAT TGTGCAATGT AAACTCCATG CCGTGGGAGT TCTTGTCCTT CAAGTGCTTG   300
ACCAATATGT TTCTGTTGGC AGAGGGAACC TGTCAACTAG TTAATAACTA GTCAGAAACT   360
AGTATAGCAG TAGACTCACT GTACGCTTGA GGCCCCTCTC TCTCTTTGCA CTGACTGTCA   420
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GAGGAGACAC CATTAAAATT GGGTCTGGCT TGATTCATGA CATACATTCG TCGTCTTGAA   600
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CAGCGTTCGT TTAGGGCGGG TAGACTGGAA TTTATTCCTT GCGCCACGGA CCAATCGCTC   960
CCTCGACGCT CTCATTCCTG CGTCGAGCTC TTTTTCCCTC GACTCTCATT GCTTGCTGGG  1020
CTGGTTCTTG AACCTCTTCA ATCGTCCTTA TCTCTTTCCC CCCATCCGGC CTGTGATTCC  1080
TATCTTTCCT TTTTTTCTTC CCTTTCTTGT TTGATCCCCC CTCCTCCCCG TCTTATCGCC  1140
TACTATCGTG ATCCCCGCCC TTCCCAATAA AGAGTAGGGC GTGTGAACAT GTCCGGGTTA  1200
ACCCTCGGGC GAGGCCCTGG GGGCGTGCGA CCGACTCAAA CCGCAACTTT TACCACCCAC  1260
CACCCGTCCG CCGATGCTGA CCGCCCAAAC AACCTCCCCC CTACCTCCTC GCAGCTGTCC  1320
GATGACTTTT CTTTCGGTTC CCCTCTGAGC CCCGCCGACT CACAGGCCCA TGACGGCCTA  1380
CTTCAGGACT CCCTCTTCCC TGAATGGGGG TCTGGTGCGC CTCGACCCGG CATTGACAGT  1440
CCGGATGAGA TGCAGAGGCA AGATCCGTTA GCGACTCAAA TATGGAAGCT CTATTCTAGG  1500
ACCAAGGCCC AGTTGCCCAA CCAGGAGCGT ATGGAAAACC TGACCTGGCG GATGATGGCG  1560
ATGAGTTTGA AACGTAAGGA GCGGGAACGT GCTCAACAGT CCATGTAGGT GTTCTCCCTC  1620
TGTAGAGGAA CGGCTGGACC CGCTCATCAT TAATTTTTTT TTGTCTGTGA AGGTTTCCTG  1680
CGAGACGCGG TAGCTGGCCC CAGTGGTATC GCTCAACTGC GCATTTCCGA CCCGCCCGTT  1740
GCCACCGGTA ACCCTCAGTC AACCGACCTG ACCGCCGACC CTATGAACCT CGACGATTTC  1800
ATCGTGCCCT TCGAATCTCC TTCGGACCAC CCCTCGCCCA GTGCCGTCAA GATTTCCGAC  1860
TCCACGGCGT CCGCGGCCAT TCCCATCAAG TCCCGGAAAG ACCAGCTGAG AGATTCTACC  1920
CCGGTGCCGG CCTCGTTCCA CCATCCGGCT CAGGATCAAC GGAAGAACAG TGAATTTGGC  1980
TACGTCCCCC GTCGCGTGCG CAAGACGAGT ATCGACGAGC GTCAATTTTT CTCACTGCAG  2040
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GTGCCGACCC GAAAGCGACC GGCCGAATCC TCGCCCCAGG TACCCCCCGT TTCCAACTCG 2100

ATGTTGGCCC ACGATCCGGA CCTCGCTTCC GGCGTGCCCG ATTATGCCTT GGACGCCCCG 2160

TCCTCGGCCT TTGGCTTCCA TCAAGGTAAC CACCATCCGG TCAATCATCA CAACCACACC 2220

TCCCCCGGGG CACCGTTTGG CTTGGATACG TTCGGCCTGG GAGATGATCC AATCTTGCCC 2280

TCCGCGGGCC CCTACCAGTC GCAATTCACC TTCTCACCCA GCGAGTCTCC GATGGCCTCC 2340

GGTCATCCGT TTGCGAACCT CTATTCGCAT ACCCCGGTGG CTTCGTCCCT CAACTCGACG 2400

GATTTCTTCT CTCCACCGCC ATCAGGCTAC CAGTCCACGG CATCCACGCC GCAGCCCACC 2460

TACGACGGGG ACCATTCCGT TTATTTCGAT ATGCCGTCGG GCGACGCGCG CACCCAGCGC 2520

CGCATTCCGA ACTATATTTC GCATCGGTCC AACTTGTCTG CTTCGCTGCA GCCTCGGTAT 2580

ATGTTCAACC AGAACAACCA TGAACAGGCC AGTTCGTCGA CGGTGCATTC GCCGAGCTAC 2640

CCCATTCCCC AGCCGCAACA TGTGGACCCC ACTCAGGTGT TGAACGCCAC CAATTACTCG 2700

ACCGGCAACT CCCACCATAC CGGCGCCATG TTTTCATTTG GAGCCGATTC AGATAACGAG 2760

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GACGAGGACG GGTTCTCGTC GGGCATGCAG TGGGATGGGC AGTTCCCGGG CTCCTTCCAT 2880

TCGCTGCCGG GCTTTGGCCC TCAACATCGC AAGCATGTTA CCATCGGGTC CACGGACATG 2940

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GCTTCGGTCA GTGAGGTGCG CAACCGAGAG CAGGACCCTC GCCGGCAGAA GATTGCGCGC 3060

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ACGTCTCATA CCTCCCCTAA TACGCCGCCC GAGTCCGCCC TGAGCAGCGC AGTTCCGTCC 3180

CGCCCGGCCA GTCCCGGGGG CAGCAAGAAC GGCGACCAAG GCAGCAACGG ACCGACCACC 3240

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GCCGCGGGAC GGTCGAATGG GGTGGTACCC ATTGCCGCCG CTCCTCCGAA GGCAGCTCCC 3600

TCCGCAGCCG CCTCCCCTAG CACGGGCCAG ACCCGCAACC CGATCCAGGC TGCCCCGAAA 3660

CGTCAACGAC GGCTGGAAAA GGCCACGGAG ATGGAAACGG ACGAGGCTAA CAAGTCCGCG 3720

GGAGGCCGAT CCAAGGTGGT GCCTCTGGCA CCCGCCATGC CACCGGCAGC AGCCAATCCG 3780

GCGAACCATA GTATTGCCGG AGGCCAAGGG GCTAGTCAGG AATGGGAGTG GTTGACGATG 3840

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ATACTGCAAC CAATGGAATC TCGCTAGACG AGAGGTGTTA GATGACGTGG CCCGCGATGC 4020

ACTTAATGAG ATACGAGGAG GTGCAATGCG TTGGTTACGC TAGTTTAATG GTAACATGAC 4080

GAGGGATATT CGCTCTGTTA TTTCGGGCTT TGATCTGTTT CAGTCTGCGA TTTAACAGCG 4140

ACTGATCCTC TGCTGTGACA ATACACAGCT TGTCTTGTGG TTCTGTTGTG GCTTTCTGTT 4200

TGTTTGGCTG ATTTGATTTA TGCTTGATAC AATCGCGTCT GTCCGGACCC CGGCCTTTGT 4260

TTTGTTTTCA GTTCTGATTC TTCACTGTTT CTGATTCTCT TGTTCATGTT TTTGATTTGT 4320

TCAAGGCTTG GGGCCGGGCA GAAGTGCGCA TCTCTGCTTT GTGTTTTCCG TCACCGTGCA 4380

TAGACGCTGT ATGTATATGC TACAGCAAGA TTCTACTTAT CCAGTCTGAG CCTGTATTCA 4440

TTGAAGTGTA GCCAGCTGTC GAATGAGCTT TTTGACGATA TTGTTTTGTT GAGTAGTCAA 4500

CAAGTAGTAT CTGTATATTC CGGAGTCTAA GTAAGACACT TGAGAATAAT GTGGAGCTTC 4560

TCGCCCTGTC ATATATCTGA ACGCTAGCCC GTAGGCCGTG AACAAGGGTG ATAAGGATAT 4620

ACTAGCCTAA TGAATTGACG TCATAGCATA TAAGCTT 4657

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 853 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(ix) FEATURE:
(A) NAME/KEY: Binding-site
(B) LOCATION: 652-676
(D) OTHER INFORMATION:/note= "DNA BINDING SITE"
(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION:1..731
(D) OTHER INFORMATION:/note= "TRUNCATED AREA WHICH IS STILL ACTIVE BUT NOT REPRESSED BY L-GLUTAM..."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Gly Leu Thr Leu Gly Arg Gly Pro Gly Gly Val Arg Pro Thr
1 5 10 15

Gln Thr Ala Thr Phe Thr Thr His His Pro Ser Ala Asp Ala Asp Arg
20 25 30

Pro Asn Asn Leu Pro Pro Thr Ser Ser Gln Leu Ser Asp Asp Phe Ser
35 40 45

Phe Gly Ser Pro Leu Ser Pro Ala Asp Ser Gln Ala His Asp Gly Leu
50 55 60

Leu Gln Asp Ser Leu Phe Pro Glu Trp Gly Ser Gly Ala Pro Arg Pro
65 70 75 80

Gly Ile Asp Ser Pro Asp Glu Met Gln Arg Gln Asp Pro Leu Ala Thr
85 90 95

Gln Ile Trp Lys Leu Tyr Ser Arg Thr Lys Ala Gln Leu Pro Asn Gln
100 105 110

Glu Arg Met Glu Asn Leu Thr Trp Arg Met Met Ala Met Ser Leu Lys
115 120 125

Arg Lys Glu Arg Glu Arg Ala Gln Gln Ser Ile Gly Ile Ala Gln Leu
130 135 140

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Arg Ile Ser Asp Pro Pro Val Ala Thr Gly Asn Pro Gln Ser Thr Asp
145                 150                 155                 160

Leu Thr Ala Asp Pro Met Asn Leu Asp Asp Phe Ile Val Pro Phe Glu
                165                 170                 175

Ser Pro Ser Asp His Pro Ser Pro Ser Ala Val Lys Ile Ser Asp Ser
            180                 185                 190

Thr Ala Ser Ala Ala Ile Pro Ile Lys Ser Arg Lys Asp Gln Leu Arg
        195                 200                 205

Asp Ser Thr Pro Val Pro Ala Ser Phe His His Pro Ala Gln Asp Gln
    210                 215                 220

Arg Lys Asn Ser Glu Phe Gly Tyr Val Pro Arg Arg Val Arg Lys Thr
225                 230                 235                 240

Ser Ile Asp Glu Arg Gln Phe Phe Ser Leu Gln Val Pro Thr Arg Lys
                245                 250                 255

Arg Pro Ala Glu Ser Ser Pro Gln Val Pro Pro Val Ser Asn Ser Met
            260                 265                 270

Leu Ala His Asp Pro Asp Leu Ala Ser Gly Val Pro Asp Tyr Ala Leu
        275                 280                 285

Asp Ala Pro Ser Ser Ala Phe Gly Phe His Gln Gly Asn His His Pro
    290                 295                 300

Val Asn His His Asn His Thr Ser Pro Gly Ala Pro Phe Gly Leu Asp
305                 310                 315                 320

Thr Phe Gly Leu Gly Asp Asp Pro Ile Leu Pro Ser Ala Gly Pro Tyr
                325                 330                 335

Gln Ser Gln Phe Thr Phe Ser Pro Ser Glu Ser Pro Met Ala Ser Gly
            340                 345                 350

His Pro Phe Ala Asn Leu Tyr Ser His Thr Pro Val Ala Ser Ser Leu
        355                 360                 365

Asn Ser Thr Asp Phe Phe Ser Pro Pro Pro Ser Gly Tyr Gln Ser Thr
    370                 375                 380

Ala Ser Thr Pro Gln Pro Thr Tyr Asp Gly Asp His Ser Val Tyr Phe
385                 390                 395                 400

Asp Met Pro Ser Gly Asp Ala Arg Thr Gln Arg Arg Ile Pro Asn Tyr
                405                 410                 415

Ile Ser His Arg Ser Asn Leu Ser Ala Ser Leu Gln Pro Arg Tyr Met
            420                 425                 430

Phe Asn Gln Asn Asn His Glu Gln Ala Ser Ser Ser Thr Val His Ser
        435                 440                 445

Pro Ser Tyr Pro Ile Pro Gln Pro Gln His Val Asp Pro Thr Gln Val
    450                 455                 460

Leu Asn Ala Thr Asn Tyr Ser Thr Gly Asn Ser His His Thr Gly Ala
465                 470                 475                 480

Met Phe Ser Phe Gly Ala Asp Ser Asp Asn Glu Asp Gly Asp Gly His
                485                 490                 495

Gln Leu Ser Glu Arg Ala Gly Leu Ala Met Pro Thr Glu Tyr Gly Asp
            500                 505                 510
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```
Glu Asp Gly Phe Ser Ser Gly Met Gln Trp Asp Gly Gln Phe Pro Gly
        515                 520                 525

Ser Phe His Ser Leu Pro Gly Phe Gly Pro Gln His Arg Lys His Val
    530                 535                 540

Thr Ile Gly Ser Thr Asp Met Met Asp Thr Pro Glu Glu Trp Asn His
545                 550                 555                 560

Gly Gly Ser Leu Gly Arg Thr His Gly Ser Val Ala Ser Val Ser Glu
                565                 570                 575

Val Arg Asn Arg Glu Gln Asp Pro Arg Arg Gln Lys Ile Ala Arg Thr
            580                 585                 590

Thr Ser Thr Pro Asn Thr Ala Gln Leu Leu Arg Gln Ser Met His Ser
        595                 600                 605

Asn Asn Asn Thr Ser His Thr Ser Pro Asn Thr Pro Pro Glu Ser Ala
    610                 615                 620

Leu Ser Ser Ala Val Pro Ser Arg Pro Ala Ser Pro Gly Gly Ser Lys
625                 630                 635                 640

Asn Gly Asp Gln Gly Ser Asn Gly Pro Thr Thr Cys Thr Asn Cys Phe
                645                 650                 655

Thr Gln Thr Thr Pro Leu Trp Arg Arg Asn Pro Glu Gly Gln Pro Leu
            660                 665                 670

Cys Asn Ala Cys Gly Leu Phe Leu Lys Leu His Gly Val Val Arg Pro
        675                 680                 685

Leu Ser Leu Lys Thr Asp Val Ile Lys Lys Arg Asn Arg Ser Ser Ala
    690                 695                 700

Asn Ser Leu Ala Val Gly Thr Ser Arg Ala Ser Lys Lys Thr Ala Arg
705                 710                 715                 720

Lys Asn Ser Val Gln Gln Ala Ser Val Thr Thr Pro Thr Ser Ser Arg
                725                 730                 735

Ala Gln Asn Gly Thr Ser Glu Ser Pro Pro Ala Gly Phe Ser Ala Ala
            740                 745                 750

Ala Gly Arg Ser Asn Gly Val Val Pro Ile Ala Ala Ala Pro Pro Lys
        755                 760                 765

Ala Ala Pro Ser Ala Ala Ala Ser Pro Ser Thr Gly Gln Thr Arg Asn
    770                 775                 780

Pro Ile Gln Ala Ala Pro Lys Arg Gln Arg Arg Leu Glu Lys Ala Thr
785                 790                 795                 800

Glu Met Glu Thr Asp Glu Ala Asn Lys Ser Ala Gly Gly Arg Ser Lys
                805                 810                 815

Val Val Pro Leu Ala Pro Ala Met Pro Pro Ala Ala Ala Asn Pro Ala
            820                 825                 830

Asn His Ser Ile Ala Gly Gly Gln Gly Ala Ser Gln Glu Trp Glu Trp
        835                 840                 845

Leu Thr Met Ser Leu
    850
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```
(2) INFORMATION FOR SEQ ID NO: 3:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 21 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: other nucleic acid
          (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGAATTCATG AGTGGCATCG C                                              21


(2) INFORMATION FOR SEQ ID NO: 4:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 22 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: other nucleic acid
          (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCTAGACTAC AAACTCATCG TC                                             22


(2) INFORMATION FOR SEQ ID NO: 5:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 23 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: other nucleic acid
          (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GAATTCCATG GTGTCCTCGT CGG                                            23


(2) INFORMATION FOR SEQ ID NO: 6:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 24 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: other nucleic acid
          (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAATTCGAGC CGTCAGTGAG GCTC                                           24


(2) INFORMATION FOR SEQ ID NO: 7:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 27 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: other nucleic acid
          (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTTGCCATTG CTGCAGGCAT CGTGGTG                                        27
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```
(2) INFORMATION FOR SEQ ID NO: 8:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 27 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCCGGGCCTC TTGCGGGCGT CCATTCC                                  27


(2) INFORMATION FOR SEQ ID NO: 9:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 32 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CATCCGTCAC GACTTAAGAT ATCAAGCCGC GC                            32


(2) INFORMATION FOR SEQ ID NO: 10:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 19 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CACAGGAAAC AGTCACGAC                                           19


(2) INFORMATION FOR SEQ ID NO: 11:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 18 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CGTTTTCCCA GTCACGAC                                            18


(2) INFORMATION FOR SEQ ID NO: 12:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 22 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION:   /desc = "OLIGONUCLEOTIDE"
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGGCCCGGTA CCCAATTCGC CC                                       22
```

(2) INFORMATION FOR SEQ ID NO: 13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GATATCGGTT TATTGTGGCC G 21

(2) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGTTTTTTCC ACCATGCGGC CGCAAGGTAC GTCAATTC 38

(2) INFORMATION FOR SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GACTTGGAGG AGTAGTTAAC GGCACATCAT TC 32

(2) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATGCGGCCGC TAACCCTCGG GCGAGGCCC 29

(2) INFORMATION FOR SEQ ID NO: 17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTAAGTCGTG ACGGATGCTT GC 22

Claims

1. A koji mold which is capable to express at least 2 times more endo- and exo-peptidases than the wild type strain *Aspergillus oryzae* CNCM I-1882.

2. A koji mold according to claim 1, which expresses at least 30 mU of endopeptidase activity, at least 30 mU of leucine-amino-peptidase activity and at least 10 mU of proline-dipeptidyl-peptidase activity per ml of supernatant when grown in a minimal medium containing 0.2% soy bean proteins.

3. A koji mold according to claim 1, which is capable to express the proteolytic activities in presence of at least 5mM L-glutamine.

4. A koji mold according to claim 1, which contains an *are*A gene which is not repressed when the mold is grown in a minimal medium containing repressive amounts of L-glutamine.

5. A koji mold according to claim 4, wherein the *are*A gene is truncated so the C-terminally truncated AREA protein remains functional but not not repressed when the mold is grown in a minimal medium containing repressive amounts of L-glutamine.

6. A koji mold according to claim 4, which has integrated multiple copies of the *are*A gene.

7. A koji mold according to claim 5, wherein the *are*A gene is operably linked to at least one regulatory sequence able to direct over-expression of the *are*A gene.

8. A koji mold according to claims 5 or 6, wherein the *are*A gene has the nucleotide sequence defined by nucleotides 1189-1604 and 1704-3480 of SEQ ID NO:1 or functional derivatives thereof due to the degeneracy of the genetic code.

9. A koji mold according to one of any preceeding claims 1-8 selected from the genus *Aspergillus, Rhizopus* or *Mucor.*

10. A koji mold according to claim 9 which is selected from strains *Aspergillus oryzae* CNCM I-1881, CNCM I-1883 and CNCM I-1884.

11. A DNA-binding protein of *Aspergillus oryzae* (AREA) having at least the amino-acid sequence from amino-acid 1 to amino-acid 731 of SEQ ID NO:2 or functional derivatives thereof.

12. A DNA molecule which comprises an *are*A gene encoding the protein according to claim 11.

13. A DNA molecule according to claim 12, which is a vector comprising the *are*A gene.

14. A DNA molecule according to claim 12, wherein the *are*A gene is operably linked to at least one regulatory sequence able to direct the expression of the said gene.

15. A DNA molecule according to claim 12, wherein the *are*A gene has at least the nucleotide sequence defined by nucleotides 1189-1604 and 1704-3480 of SEQ ID NO:1 or functional derivatives thereof due to the degeneracy of the genetic code.

16. A method for over-producing proteolytic enzymes, comprising cultivating a koji mold according to claims 1-10 in a suitable growth medium under conditions that the mold expresses enzymes, and optionally isolating the enzymes in the form of a concentrate.

17. Use of the koji mold according to claim 1-10 to hydrolyse protein-containing materials.

18. Use according to claim 17, in combination with an enzyme and/or a microoganisme providing a prolidase activity.

19. Use according to claims 17 or 18, wherein the protein-containing materials comprise at least 5mM of L-glutamine.

20. A food product comprising a protein hydrolysate obtainable by fermentation with a koji mold according to claims 1-

10 of a material comprising proteins and at least 5mM of L-glutamine.

Figure 1

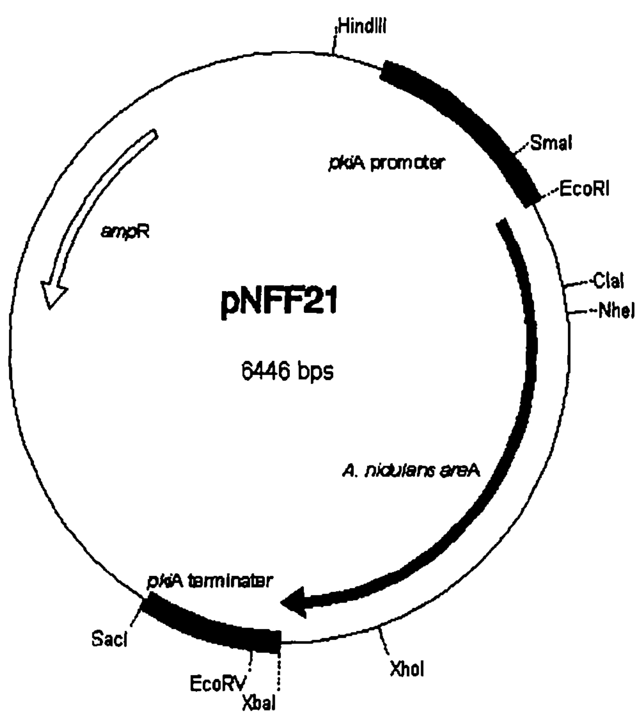

HindIII
SmaI
EcoRI
pkiA promoter
ampR
pNFF21
6446 bps
ClaI
NheI
A. nidulans areA
pkiA terminater
SacI
EcoRV
XbaI
XhoI

Figure 2

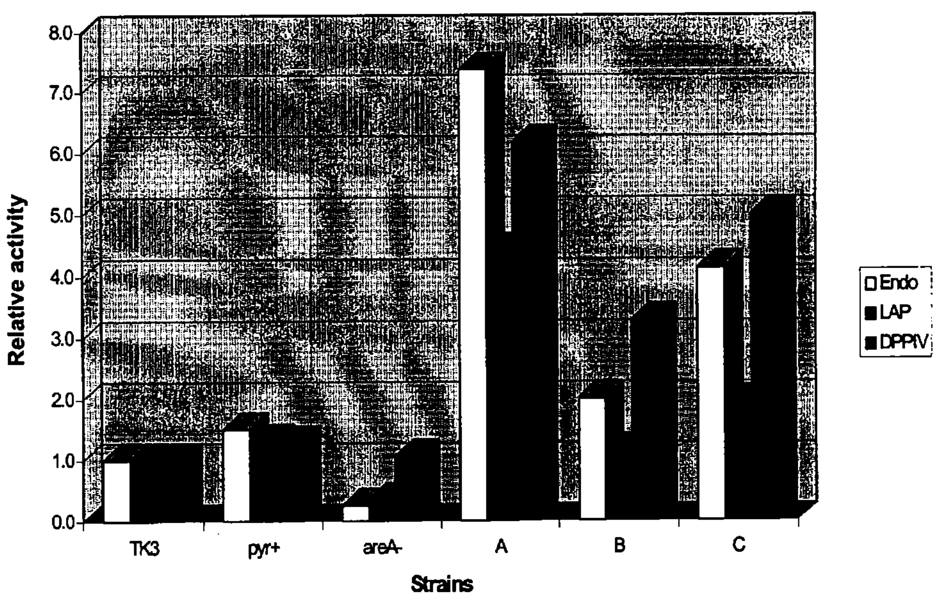

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Relative activity
TK3
pyr+
areA-
A
B
C
Strains
Endo
LAP
DPPIV

Figure 3

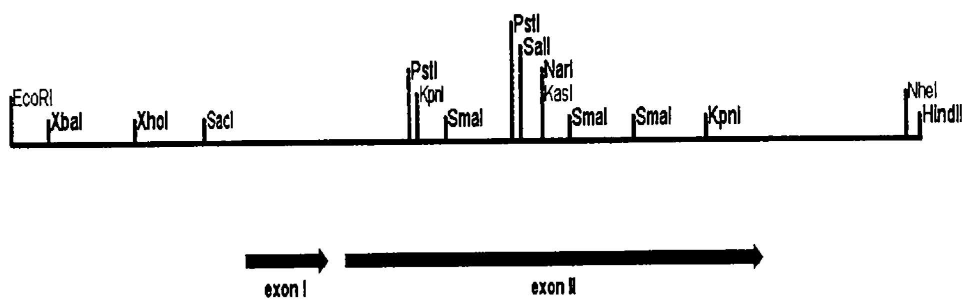

EcoRI
XbaI
XhoI
SacI
PstI
KpnI
SmaI
PstI
SalI
NarI
KasI
SmaI
SmaI
KpnI
NheI
HindIII
exon I
exon II

Figure 4

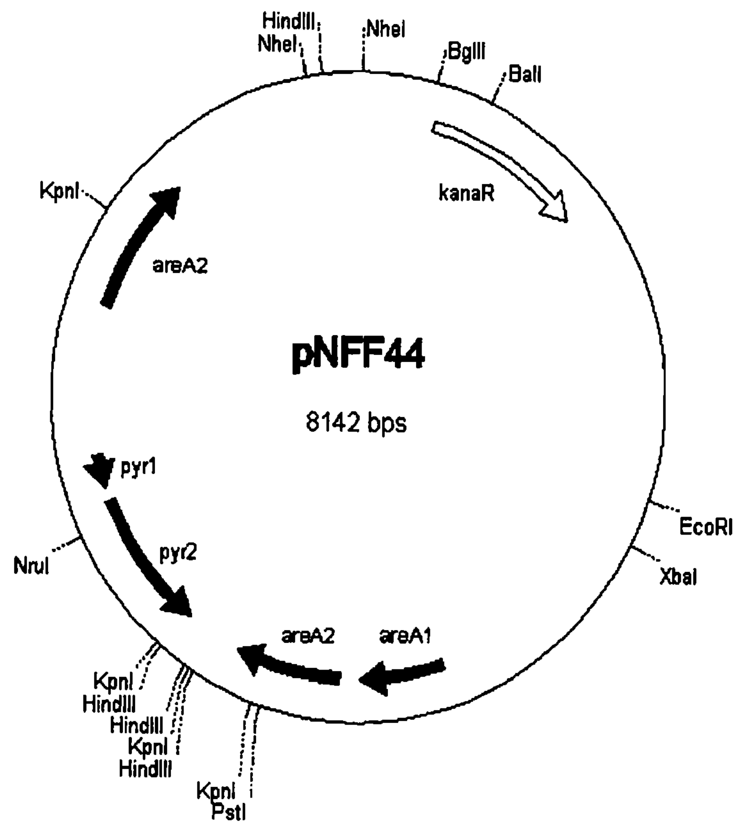
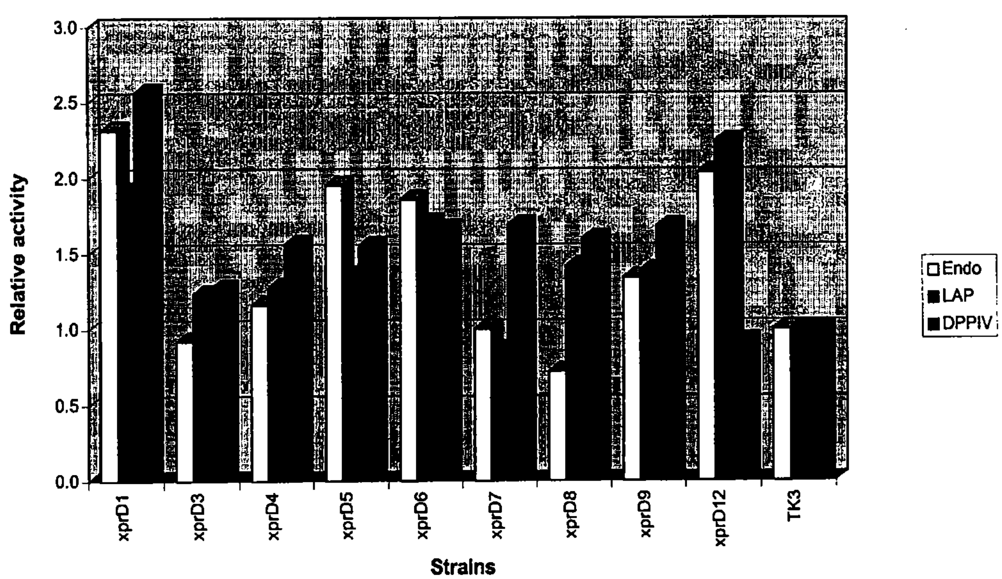

HindIII
NheI
NheI
BglII
BalI
kanaR
KpnI
areA2
pNFF44
8142 bps
pyr1
pyr2
NruI
EcoRI
XbaI
areA2
areA1
KpnI
HindIII
HindIII
KpnI
HindIII
KpnI
PstI

Figure 5

```
WT       CATCCGTCACGACT.CCGACATCAAGCCGCGC
         ||||||||||||||    || ||||||||||||
mutant   CATCCGTCACGACTTAAGATATCAAGCCGCGC
                                 EcoRV
```

Figure 6


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Relative activity
xprD1
xprD3
xprD4
xprD5
xprD6
xprD7
xprD8
xprD9
xprD12
TK3
Strains
Endo
LAP
DPPIV

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 11 1378

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,X	KUDLA B ET AL: "THE REGULATORY GENE AREA MEDIATING NITROGEN METABOLITE REPRESSION IN ASPERGILLUS NIDULANS. MUTATIONS AFFECTING SPECIFICITY OF GENE ACTIVATION ALTER A LOOP RESIDUE OF A PUTATIVE ZINC FINGER" EMBO JOURNAL, vol. 9, no. 5, April 1990, pages 1355-1364, XP000615427	1-15	C12N15/31 C12N1/15 C07K14/38 C12N9/62 A23J3/16 A23J3/18 C12P21/06
Y	* the whole document * especially figure 9	16-20	
X	M. STANKOVICH ET AL: "C-terminal truncation of the transcriptional activator encoded by areA in Aspergillus nidulans results in both loss-of-function and gain of function phenotypes" MOLECULAR MICROBIOLOGY, vol. 7, no. 1, 1993, pages 81-87, XP002048815	1-15	
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Y	WO 95 35385 A (NOVONORDISK AS ;CHRISTENSEN TOVE (DK); HYNES MICHAEL J (AU)) * the whole document * especially page 2, paragraph 1	16-20	C07K C12N
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	2 December 1997	Van der Schaal, C

CATEGORY OF CITED DOCUMENTS

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EUROPEAN SEARCH REPORT

Application Number
EP 97 11 1378

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	BIOLOGICAL ABSTRACTS, vol. 97, Philadelphia, PA, US; abstract no. 545238, JARAI G ET AL: "Nitrogen, carbon, and pH regulation of extracellular acidic proteases of Aspergillus niger." XP002048816 * abstract * & CURRENT GENETICS 26 (3). 1994. 238-244. ISSN: 0172-8083, ---	16-20	
Y	DATABASE WPI Section Ch, Week 9527 Derwent Publications Ltd., London, GB; Class D13, AN 95-202831 XP002048808 & JP 07 115 969 A (ASAHI KASEI KOGYO KK), 9 May 1995 * abstract * ---	18	
D,Y	EP 0 417 481 A (NESTLE SA) * the whole document * -----	20	TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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